

## **Supplementary Information**

### **Structural and molecular rationale for the diversification of resistance mediated by the Antibiotic\_NAT family**

Peter J Stogios<sup>1,#</sup>, Emily Bordeleau<sup>2,#</sup>, Zhiyu Xu<sup>1</sup>, Tatiana Skarina<sup>1</sup>, Elena Evdokimova<sup>1</sup>, Sommer Chou<sup>2</sup>, Luke Diorio-Toth<sup>3</sup>, Alaric W. D'Souza<sup>3</sup>, Sanket Patel<sup>3</sup>, Gautam Dantas<sup>3,4,5,6</sup>, Gerard D. Wright<sup>2</sup> and Alexei Savchenko<sup>1,7,8,\*</sup>

<sup>1</sup>Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Canada

<sup>2</sup>David Braley Centre for Antibiotics Discovery M.G. DeGroot Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada

<sup>3</sup>The Edison Family Center for Genome Sciences & Systems Biology, Washington University School of Medicine, St. Louis, MO, USA, 63108

<sup>4</sup>Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA, 63108

<sup>5</sup>Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, MO, USA, 63130

<sup>6</sup>Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, USA, 63108

<sup>7</sup>Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Canada, T2N 4N1.

<sup>8</sup>Center for Structural Genomics of Infectious Diseases (CSGID), University of Calgary, Calgary, Canada, T2N 4N1.

\*to whom correspondence should be addressed: Alexei Savchenko, Department of Immunology and Infectious Diseases, Health Research Innovation Centre, 3330 Hospital Drive NW, University of Calgary, Calgary, AB, T2N 4N1, Canada. Tel: +1-403-210-7980; Email: alexei.savchenko@ucalgary.ca

#These authors contributed equally

**Supplementary Figure 1 – Sequence alignment of the Antibiotic\_NAT family.**

Sequences are grouped and shaded according to phylogenetic reconstruction in Figure 1. Includes meta-AAC's, AAC(3) enzymes from clinical isolates, and sequences identified through BLAST searches of NCBI. Darker color or shading of amino acids indicates higher conservation. Major and minor subdomains are indicated with solid and dashed black lines, respectively, above the sequence alignment.

major subdomain

Table with 5 columns (1, 10, 20, 30, 40, 50) and 50 rows of sequence data. Includes labels for Group 1, Group 2, Group 3, and Group 4. The sequences are aligned across the major subdomain.

minor subdomain

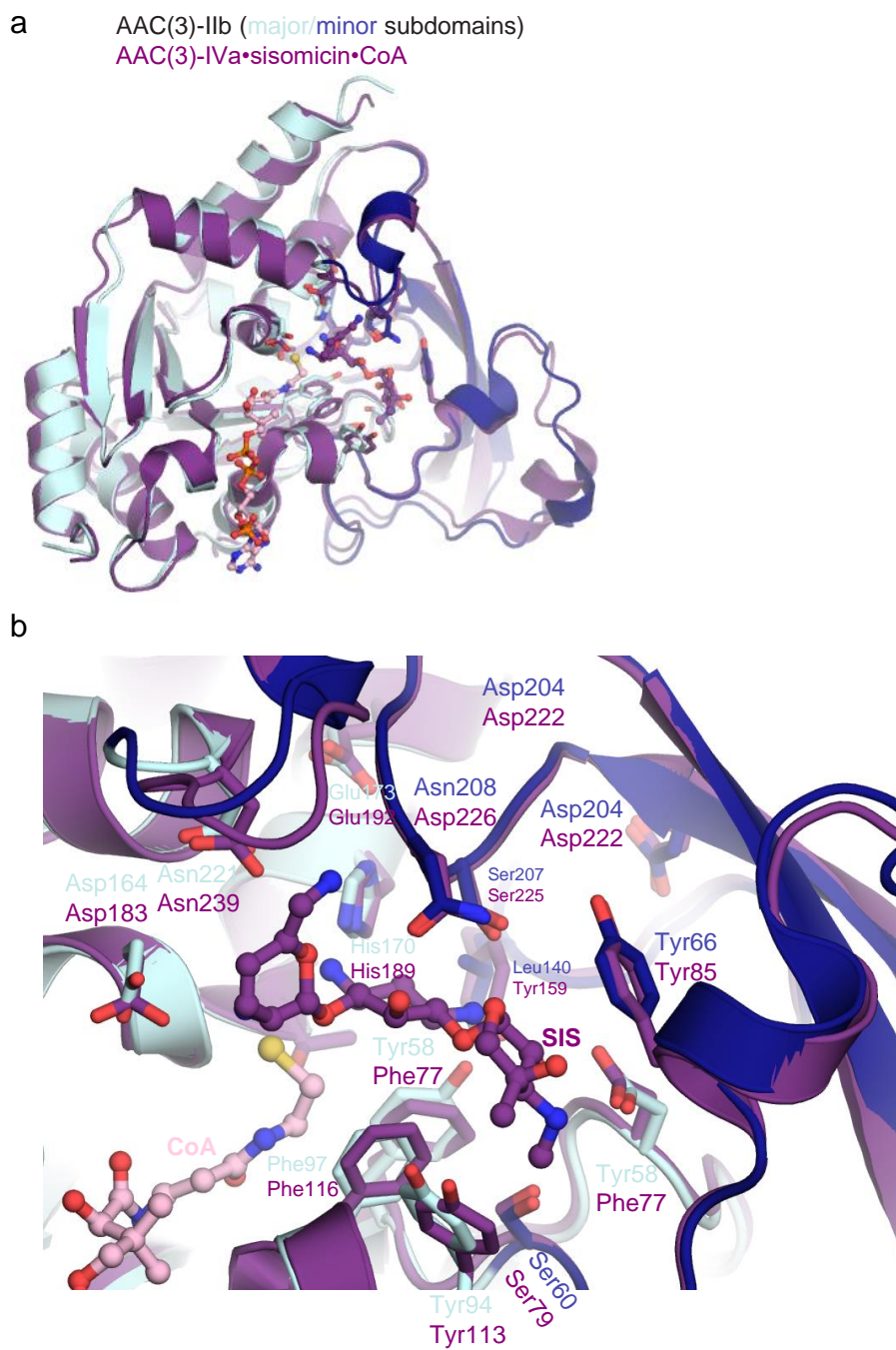
Table with 5 columns (60, 70, 80, 90) and 50 rows of sequence data. Includes labels for Group 1, Group 2, Group 3, and Group 4. The sequences are aligned across the minor subdomain.



	220	230	240	250	260
AAC_3_Iva_WP_063840268.1	EKSLQR	EGPVGHAFAR	LRSRDIVATA	LGDRPLIF	LHPPEAGCECDAARQSIG
S_melliloti_WP_027993499.1	RELQLR	EGPVGHAFAR	LRSRDIVRIAS	DRIARDPLVF	LHPPEAACCECDAARQSIG
Rhodomicrobium_WP_088346198.1	ARLQSR	EGPVGHAFAR	LRSRDIVRAAI	GHIADPEPLVF	LHRGAGHCPACDAARQSTSG
metaAAC0016_AIA14255.1	DAERQA	EGPVGHAFAR	LADARDIVAVAE	ERIVADPLVF	LHPPEAGCPCVEARQSTVSG
metaAAC0074_AMP57367.1	ARLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Sphingomonas_sp._WP_029724114.1	AEELQR	EGPVGHAFAR	LRSRDIVRVAV	PRVQADPLVF	LHGSEGCCECDARQSLSR
metaAAC0018_AIA14757.1	AEELQR	EGPVGHAFAR	LRSRDIVRVAV	PRVQADPLVF	LHGADQGCCECDARQSLSR
Allotkutneria_albata_WP_03043120.1	DGAQR	EGPVGHAFAR	LRSRDIVRVAV	PRVQADPLVF	CAEGSGCEDCDARQSLSR
Archangium_gephyra_WP_047855546.1	ARLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Cystobacter_fuscus_WP_043428585.1	ARLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Myxococcus_fulvus_SEU16843.1	ARLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Sorangium_cellulosum_KYF78036.1	GRLOQR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Sorangium_cellulosum_WP_04498544.1	ERLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Stigmatella_aur._WP_013378336.1	PRLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Sandaracinus_amiI._WP_053233315.1	ARLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0033_AIA17596.1	AKLQSR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0022_AIA16407.1	AQSMQR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Ixa_WP_063840269.1	SAIGLR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_VIIa_WP_063840272.1	AAIGLR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Xa_WP_012377682.1	AAIGLR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_VIIa_WP_063856943.1	AAIGLR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_IIIa_WP_063840261.1	AQGGGT	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0008_AIA12232.1	AQKTS	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
P_aeruginosa_WP_023911614.1	AEERGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0030_AMP48516.1	AQKGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0071_AMP57363.1	ADPKIV	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_IIb_WP_088170001.1	ASQGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_IIc_WP_063840263.1	AGGRQR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Bossea_lupini_WP_091829703.1	AAEGGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
P_aeruginosa_WP_042854441.1	AAEGGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Rhizobium_etLi_WP_039618492.1	AGQGA	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Devosia_insulae_WP_069908639.1	ATGRGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Uncultured_AAL92107.1	ATGRGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0038_AIA18843.1	ATGRGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Uncultured_AAL92107.1	ATGRGR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Inquillinus_limosus_WP_026870780.1	AAEGGA	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0032_AIA17598.1	AAANNR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_-IIa_WP_163592000.1	KLGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0043_ACT97599.1	KLGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Uncultured_AGC09640.1	EKTNNR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Via_6BC2_WP_063840273.1	ARTRVA	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Massilia_alk._WP_027865365.1	ALGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Niveispirillum_cy._WP_102114653.1	GKRRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Hydrogenophaga_sp._WP_086125356.1	SEGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0034_AIA17583.1	RLGRCR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0035_AIA17960.1	RLGRCR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0029_AMP47836.1	SEGHAR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
metaAAC0070_AMP48506.1	SEGHAR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Ensifer_sp._KW34869.1	LLGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Ifg_WP_012695485.1	ELRRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Iib_WP_033147097.1	ELRRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Sinorhizobium_sp._GL2_KSV77101.1	ATSRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
Sinorhizobium_WP_058327775.1	KLGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Iic_CAA38525.1	KLGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
AAC_3_Iia_WP_063840264.1	KLGRHR	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
BA2930_3E4F	QKTTVF	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
BsYokD_ZNYG	AEINMK	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK
SrFrbF_3SMA	TRPGRS	EGPVGHAFAR	LRSRDIVVAIE	EYSMPLVF	LHPVAGGCEDCDARQTAGAAPFK

AAC_3_Iva_WP_063840268.1	....
S_melliloti_WP_027993499.1	GGGQF
Rhodomicrobium_WP_088346198.1	....
metaAAC0016_AIA14255.1	MG
metaAAC0074_AMP57367.1	TG
Sphingomonas_sp._WP_029724114.1	....
metaAAC0018_AIA14757.1	....
Allotkutneria_albata_WP_03043120.1	....
Archangium_gephyra_WP_047855546.1	TGPA
Cystobacter_fuscus_WP_043428585.1	....
Myxococcus_fulvus_SEU16843.1	....
Sorangium_cellulosum_KYF78036.1	HA
Sorangium_cellulosum_WP_04498544.1	HA
Stigmatella_aur._WP_013378336.1	....
Sandaracinus_amiI._WP_053233315.1	....
metaAAC0033_AIA17596.1	....
metaAAC0022_AIA16407.1	....
AAC_3_Ixa_WP_063840269.1	....
AAC_3_VIIa_WP_063840272.1	....
AAC_3_Xa_WP_012377682.1	....
AAC_3_VIIa_WP_063856943.1	....
AAC_3_IIIa_WP_063840261.1	....
metaAAC0008_AIA12232.1	....
P_aeruginosa_WP_023911614.1	....
metaAAC0030_AMP48516.1	....
metaAAC0071_AMP57363.1	....
AAC_3_IIb_WP_088170001.1	....
AAC_3_IIc_WP_063840263.1	....
Bossea_lupini_WP_091829703.1	....
P_aeruginosa_WP_042854441.1	....
Rhizobium_etLi_WP_039618492.1	....
Devosia_insulae_WP_069908639.1	....
Uncultured_AAL92107.1	....
metaAAC0038_AIA18843.1	....
Uncultured_AAL92107.1	....
Inquillinus_limosus_WP_026870780.1	....
metaAAC0032_AIA17598.1	....
AAC_3_-IIa_WP_163592000.1	....
metaAAC0043_ACT97599.1	....
Uncultured_AGC09640.1	....
AAC_3_Via_6BC2_WP_063840273.1	....
Massilia_alk._WP_027865365.1	....
Niveispirillum_cy._WP_102114653.1	....
Hydrogenophaga_sp._WP_086125356.1	....
metaAAC0034_AIA17583.1	....
metaAAC0035_AIA17960.1	....
metaAAC0029_AMP47836.1	....
metaAAC0070_AMP48506.1	....
Ensifer_sp._KW34869.1	....
AAC_3_Ifg_WP_012695485.1	....
AAC_3_Iib_WP_033147097.1	....
Sinorhizobium_sp._GL2_KSV77101.1	....
Sinorhizobium_WP_058327775.1	....
AAC_3_Iic_CAA38525.1	....
AAC_3_Iia_WP_063840264.1	....
BA2930_3E4F	....
BsYokD_ZNYG	....
SrFrbF_3SMA	....

## Supplementary Figure 2 – Structural analysis of Group 4 AAC(3)-IIb vs. AAC(3)-VIa.



- a) Superposition of overall structures. b) Zoom of active sites, sisomicin and CoA are shown in ball-and-stick representation.

**Supplementary Table 1. Aminoglycoside susceptibility of *E. coli* harboring Antibiotic\_NAT genes**

*E. coli* BW25113  $\Delta tolC\Delta bamB$  expressing individual Antibiotic\_NAT genes under the control of the  $P_{bla}$  promoter in vector pGDP3. Shown are MIC values in  $\mu\text{g/mL}$  grouped and coloured as in Figure 2. Raw data used to derive this table is shown in **Supplementary Data 1**.

**Group 1**

	Control	Meta-AAC0016	Meta-AAC0018	HMB0022	HMB00033	AAC(3)-IVa
<b>APR</b>	4	> 256	> 256	> 256	> 256	> 512
<b>TOB</b>	0.5	> 256	> 256	> 256	> 256	$\geq 64$
<b>GEN</b>	0.25-0.5	$\geq 256$	128-256	64	$\geq 256$	> 512
<b>KAN</b>	2	128-256	64-128	8	32	64
<b>AMI</b>	1-2	1-2	1	1-2	1	1
<b>NEO</b>	1	64	128	8-16	64-128	64-128
<b>PAR</b>	2	> 256	128	16	64-128	128-256

**Group 2**

	AAC(3)-VIIa	AAC(3)-VIIIa	AAC(3)-IXa	AAC(3)-Xa
<b>APR</b>	ND	ND	4	2-4
<b>TOB</b>	ND	ND	$\leq 0.5$	16-32
<b>GEN</b>	ND	ND	$\leq 0.5$	8
<b>KAN</b>	ND	ND	2	64-128
<b>AMI</b>	ND	ND	1	1
<b>NEO</b>	ND	ND	1	1
<b>PAR</b>	ND	ND	2	4

### Group 3

	Meta-AAC0008	Meta-AAC0030	Meta-AAC0038	Meta-AAC0071	AAC(3)-IIIa	AAC(3)-IIIc	AAC(3)-IIIb
APR	16-32	16	32-64	4-8	8-16	ND	8
TOB	> 256	> 256	> 256	> 256	> 256	ND	> 256
GEN	> 256	> 256	> 256	> 256	> 256	ND	> 256
KAN	> 256	> 256	> 256	> 256	> 256	ND	> 256
AMI	1	1-2	1-2	1-2	1	ND	0.5 – 1
NEO	> 256	64-128	256	1	128-256	ND	256
PAR	> 256	> 256	> 256	4	> 256	ND	> 256

### Group 4

	Meta-AAC0029	Meta-AAC0032	Meta-AAC0035	Meta-AAC0043	Meta-AAC0070	AAC(3)-IIa	AAC(3)-IIb	AAC(3)-IIc	AAC(3)-VIa	Meta-AAC0034
APR	4	2-4	8	4	ND	8	16-32	2	4	8
TOB	64	4-8	> 256	32-64	ND	> 64	> 256	64 - 128	8	> 256
GEN	> 64	64	> 256	≥ 256	ND	> 64	> 256	> 256	≥ 256	> 64
KAN	16	16-32	≥ 256	16	ND	>64	> 256	32	16	> 256
AMI	0.5-1	1-2	1	1-2	ND	2	1	0.5-1	1-2	1-2
NEO	≤ 1	1	1	1	ND	1	0.5-1	0.5-1	≤ 1	≤ 1
PAR	1	2	2	2-4	ND	2-4	1-2	0.5-1	2	1

ND = No data.